Dynamics of the Internal and External Hydration of Globular Proteins

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Long recognized as a major determinant of protein folding and stability,[1-4] the water–protein interaction has recently been directly implicated in the recognition, binding, and catalytic events of protein function.[5-10] Although crucial for protein function, the dynamic aspects of the water–protein interaction remain poorly understood; in particular, the residence times of protein-associated water molecules have proved elusive. Here we show, for the first time, that the frequency dependence (dispersion) of the water ^{17}O spin relaxation rate provides reliable information about the residence times of water molecules buried inside the protein as well as of the water molecules in contact with the protein surface. By demonstrating that the low-frequency relaxation enhancement is due to a few internal water molecules, with residence times in the range 10^{-8}–10^{-6} s, our results resolve a long-standing interpretational controversy[11-14] and open up new possibilities for using internal water molecules in proteins as probes of unfolding dynamics and biological function.

Our approach is based on two critical considerations. First, we study the spin relaxation of the ^{17}O isotope in water, thereby avoiding the complications of cross-relaxation[15] and exchange with labile protein hydrogens[16] that complicate the interpretation of ^1H and ^2H relaxation data. Second, we focus on two small, compactly folded, globular proteins, the 58-residue bovine pancreatic trypsin inhibitor (BPTI) and the 76-residue ubiquitin, which differ in one important respect: BPTI contains four internal water molecules[17] (Figure 1), ubiquitin none.[18] These proteins should provide a decisive test of the hypothesis that the relaxation dispersion is due to internal water molecules.

Since the internal water molecules of BPTI are extensively hydrogen-bonded to main-chain atoms,[17] they can have little orientational freedom and should therefore reorient along with the protein molecule, a factor of 2000 more slowly than in bulk water. The spin relaxation rate of the internal waters is then enhanced by the same factor, provided that their residence times, τ_{res}, are longer than the rotational correlation time of the protein (6 ns). This internal-water relaxation enhancement can be transferred to the observed bulk water ^{17}O resonance only if τ_{res} is shorter than the internal-water spin relaxation time (*ca.* 4 μs). If this is the case, the four internal waters of BPTI and the *ca.* 2400 external waters (per protein molecule) in our sample should make comparable contributions to the ^{17}O relaxation rate below the dispersion frequency. For ubiquitin, which lacks internal water, only a small, frequency-independent relaxation enhancement, due to mobile water molecules interacting with the protein surface, should be seen. These expectations are fully borne out by the measured ^{17}O relaxation dispersions[19] (Figure 2). The solid curves resulted from fits of the parameters α, β, and τ_R in the theoretical expression[20,21]

$$R_1 = R_{bulk} + \alpha + \beta\tau_R[0.2/(1 + \omega_0^2\tau_R^2) + 0.8/(1 + 4\omega_0^2\tau_R^2)]$$

with $\omega_0/(2\pi)$ the Larmor frequency and τ_R the protein rotational correlation time (6 ns for both proteins).

The magnitude of the relaxation dispersion step (β) essentially yields the sum of the squares of the generalized orientational order parameter,[20] A, of all water molecules with $\tau_{res} > 6$ ns. While it could be argued[21] that the dispersion is due to a few hundred weakly ordered ($A \ll 1$), but long-lived ($\tau_{res} > 6$ ns), surface waters, such an interpretation is clearly ruled out by the insignificant relaxation dispersion for ubiquitin, which differs little from BPTI in terms of surface properties.[22] The virtual absence of a relaxation dispersion for ubiquitin demonstrates that only internal water molecules are sufficiently long-lived to sense the protein rotation. The BPTI dispersion can be accounted for by two highly ordered ($A \approx 1$) and one less ordered ($A \approx 0.5$) internal water molecule. The fourth internal water, presumably the isolated, deeply buried W122 (Figure 1), might exchange too slowly ($\tau_{res} > 4$ μs) to contribute to the ^{17}O relaxation. The finding that at least some of the completely buried water molecules in BPTI exchange on the time scale 6 ns–4 μs lowers the most recent[23] upper bound on τ_{res} for these water molecules by a factor of 5000 and suggests that local unfolding of the dominant protein conformation occurs on a submicrosecond time scale. The very weak ubiquitin dispersion can be accounted for by a single, moderately ordered ($A \approx 0.5$) water molecule, presumably W28 (ref 18), residing in a surface pocket with a hydrogen-bonding pattern similar to that of W111 at the mouth of the pore-like cavity in BPTI (Figure 1).

At high frequencies, the ^{17}O relaxation excess (α) is essentially a measure of the slowing down of water rotation at the protein surface. Assuming that the protein-induced perturbation is confined to the ca. 230 water molecules in contact with the BPTI surface,[24] we obtain an average (second-rank) rotational correlation time of 20 ps, as compared to 3.1 ps in bulk D_2O. A nearly identical result is obtained for ubiquitin. Since rotation

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(19) Recombinant bovine pancreatic trypsin inhibitor (MW 6500, 99.5% purity) was obtained from Novo Nordisk A/S and bovine erythrocyte ubiquitin (MW 8560, ca. 99% purity from gel electrophoresis) from Sigma. Protein solutions were made from heavy water enriched in ^{17}O. Protein concentrations were determined by amino acid analysis. The longitudinal ^{17}O relaxation rate, R_1, was measured at nine magnetic field strengths in the range 0.45–14.1 T using four different spectrometers.

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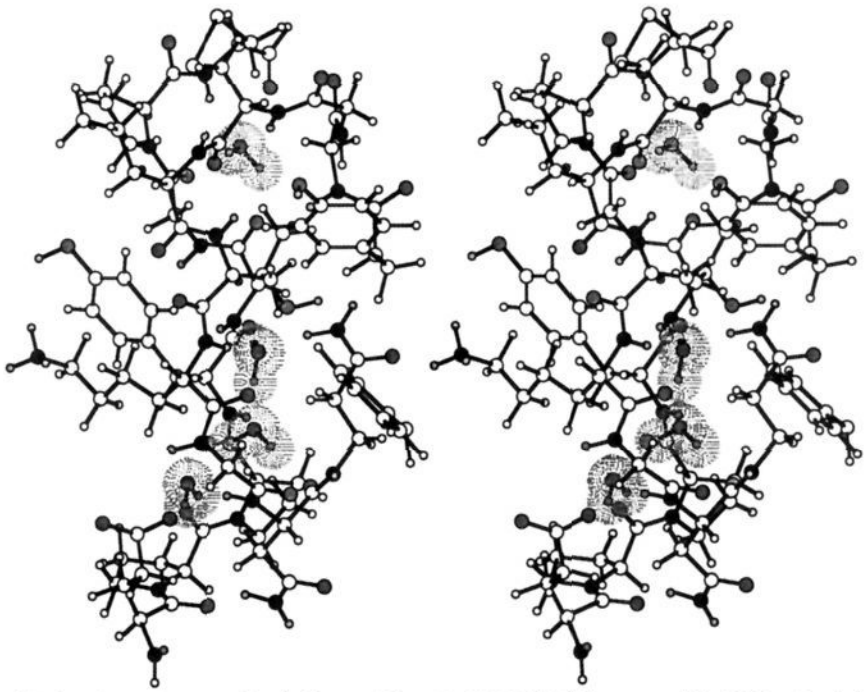

Figure 1. Stereoview of the four internal water molecules in crystal form II of BPTI.[17] (Only a part of the BPTI molecule is shown.) The dot surfaces of the water atoms correspond to 50% of the van der Waals radii. At the top is the isolated internal water molecule, W122, deeply buried in a small cavity near the Cys14–Cys38 disulfide bond. Further down is the chain of three internal water molecules, W111–W113, occupying a pore-like cavity with W111 at its mouth (bottom) and W113 deeply buried, ca. 7 Å away from W122.

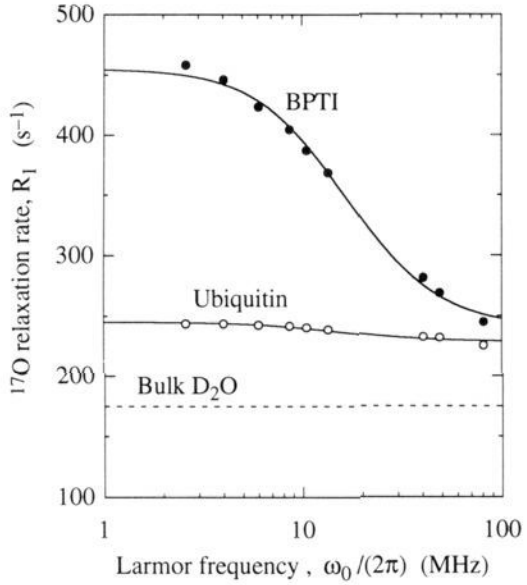

Figure 2. Water ^{17}O relaxation dispersion in D_2O solutions (27 °C) of BPTI (10.4 wt %, pD 3.40) and ubiquitin (10.2 wt %, pD 3.35). The estimated error bars are of similar size as the data symbols.

and translation of water molecules are both rate-limited by hydrogen-bond disruption, the average residence time of surface waters should be similar to the (first-rank) rotational correlation time, i.e., ca. 60 ps. Because this is much less than the protein rotational correlation time, surface waters do not contribute to the relaxation dispersion. Our picture of a highly dynamic hydration layer is consistent with recent findings from multidimensional NMR spectroscopy[25,26] and molecular dynamics simulations,[27,28] but deviates strongly from the current interpretation of dielectric relaxation data.[29,30]

By demonstrating that the high-frequency (α) and low-frequency (β) relaxation enhancements are due to two different classes of hydration water (external and internal, respectively), the present results resolve the long-standing controversy[11-14] over the origin of the relaxation dispersion of water nuclei in protein solutions. Furthermore, they shed new light on the hydration and unfolding dynamics of globular proteins and should help to clarify the molecular basis of image contrast in clinical magnetic resonance applications.

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